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(54) Title: ALZHEIMER'S PEPTIDE

(57) Abstract

The invention relates to a mammal which comprises cells which contain a transgene, the transgene comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for an Aβ peptide. A method of screening a compound for an effect on a phenotype mediated by expression of a transgenic Aβ peptide in the brain of a mammal is also disclosed. The method comprises administering the compound to a mammal expressing the transgenic Aβ peptide, and observing whether an effect on a phenotype results.

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ALZHEIMER'S PEPTIDE

BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive deterioration of memory and cognition. Prominent histopathological features of this disease include the extracellular deposition of amyloid and the accumulation of intracellular neurofibrillary tangles(1). The principal underlying cellular features of AD are the degeneration and eventual loss of neuronal cells. This often profound degeneration affects many types of neurons and may account for the numerous neurological deficits that patients afflicted with the disease encounter. The most notable degeneration occurs in the hippocampus, cerebral cortex, and amygdala(2), regions of the brain that play a major role in memory, cognition, and behavior.

Although the amyloid deposits have been the focus of much attention, their role in the pathophysiology of AD remains unclear. Amyloid deposits can occur as diffuse aggregates or as dense deposits that, together with degenerating neuritic structures, are collectively referred to as senile plaques. The principal protein component of these plaques is a 42-amino acid peptide called β-amyloid or Aβ(3,4) that is derived from a larger transmembrane protein, the Aβ precursor protein (APP), which can exist as several different isoforms(5-7).

The generation of Aβ from APP has been shown to be a normal processing event(8-10), and Aβ can be detected in the cerebrospinal fluid and plasma of both normal and AD patients(9,11). However, the process by which Aβ accumulates in the diseased brain is unknown, although mutations in the *APP* gene linked to familial AD may alter its processing and lead to higher levels of the Aβ peptide. In fact, recent *in vitro* studies have shown that cells transfected with different mutated forms of *APP* accumulate at least a six-fold higher level of Aβ compared to cells transfected with wild-type *APP*(12,13).

Several groups have shown that the endosomal/lysosomal pathway is a major processing pathway that leads to potential Aβ precursors(8-10). Despite these recent revelations concerning the processing events that may result in the formation of the Aβ peptide(8-10), it is still unclear whether the high levels of accumulation of this peptide within the brains of individuals with AD represents a cause or a consequence of the neuronal damage. To address this issue, *in vitro* studies have been employed to analyze the toxicity of this peptide. While some investigators have found that the addition of synthetic Aβ peptides as well as various fragments of APP can be cytotoxic to cells in culture, others have been unable to reproduce these findings (reviewed in refs 14,15). Similarly, attempts to study this matter *in vivo* also have yielded conflicting results in that some groups have confirmed the neurotoxicity of Aβ *in vivo*, whereas others have not(15).

Another approach designed to investigate the toxicity of Aβ *in vivo* has involved the generation of transgenic animals. Transgenic mice that expressed the human Aβ peptide(16-18), the C-terminal 100 amino acids of APP(19-22), a full-length cDNA for human APP(23,24), or the entire *APP* gene(25,26) itself have been generated under the control of various transcriptional promoter elements. These mice have not yet been reported to

develop any significant pathology, perhaps due to technical reasons such as the selection of an inappropriate promoter, low expression levels, the inability of mice to process the human precursor molecule, or inability to distinguish its effect from the genetic background effects of the host mouse strain.

Thus, there remains a need for a transgenic animal model for AD and other diseases associated with Aβ peptide expression.

SUMMARY OF THE INVENTION

The invention relates to a recombinant DNA and a transgene, each comprising a promoter operably linked to a DNA sequence coding for a β-amyloid peptide, Aβ, and a host cell or whole mammal containing the recombinant DNA or transgene. The Aβ coding sequence can be obtained from the same or different species as the cell or mammal species into which it is added. Preferably, the host species is a mouse.

The Aβ peptide or Aβ is a principal protein component of senile plaques observed in the brain of human patients having Alzheimer's disease. In humans and mice, Aβ is a 42-amino acid peptide (3,4; Yamada et al., 1987, *Biochem. Biophys. Res. Commun.* 149, 665-671) derived from a larger transmembrane protein, the Aβ precursor protein (APP), which exists as several different isoforms (5,6,7). Deposits of the Aβ peptide have been implicated in AD, Down's Syndrome, and HCHWA-D.

A nucleic acid sequence consisting essentially of a coding sequence for an Aβ peptide means essentially only the nucleic acid sequence that encodes the amino acid sequence of an Aβ peptide. For example, a nucleic acid sequence consisting essentially of a coding sequence for a mouse Aβ sequence is shown in Figure 9. The nucleic acid can be, e.g., RNA and/or DNA, and it can comprise degenerate sequences, preferred codons, etc.

A nucleic acid sequence coding for an Aβ peptide means a nucleic sequence which, when expressed by a cell, results in the production of an Aβ peptide. For example, such a nucleic acid coding sequence can be the complete coding sequence of APP or a fragment thereof, e.g., a 42-amino acid sequence encoding Aβ, or the carboxyl terminus of the APP gene. See, e.g., Kammensheidt et al., Proc. Natl. Acad, Sci., 89, 10857-10861 (1992). A recombinant nucleic acid comprising a nucleic acid sequence coding for an Aβ peptide is, therefore, any recombinant nucleic acid which can be used to express Aβ, excluding the complete and normal APP gene.

As used here, a "mouse Aβ peptide" denotes a peptide having an amino acid sequence native to mouse tissues. See, e.g., Yamada et al., Biochem. Biophys. Res. Commun., 149, 665-671 (1987). A nucleic acid sequence coding for such a peptide includes, degenerate sequences and preferred codons, as well. It is understood that naturally-occurring allelic variations exist in the Aβ peptide and occur from individual to individual. These variations include amino acid differences, e.g., substitutions, deletions, insertions, or inversions, as well as nucleic acid differences, e.g., substitutions, deletions, insertions, or inversions. Such sequences are, therefore, mouse Aβ peptides.

A mutant mammal Aβ peptide sequence can also be used according to the present invention. Such a mutant sequence, for example, can be a non-naturally occurring sequence or a sequence which copies a mutation already found in nature. For example, there is a human disease, hereditary cerebral hemorrhage with amyloidosis Dutch type (HCHWA-D), characterized clinically by cerebral hemorrhage and recurrent strokes. In this disease, Aβ peptide deposition is found predominantly in cerebral blood vessel walls and, to a lesser extent, in the neuropil. A mutation at position 22 of Aβ, resulting in the replacement of glutamic acid with glutamine, has been

identified that is associated with the disease. For expression in a mouse, the human HCHWA-D sequence encoding the Aβ peptide can be used directly, or a derivative sequence can be made in which glutamic acid is replaced with glutamine in the mouse Aβ peptide at corresponding position 220. See Figure 9. A mutant sequence can also be identified by mutagenesis, either in whole animals or cell culture, using conventional technology. For example, mutagenized Aβ sequences can be transformed into cells, and the transformed cells can be screened for overexpression or aberrant expression of the Aβ peptide, e.g., by immunoassay.

The source of the nucleic acid coding sequence can be a natural or mutant Aβ sequence obtained from a mammal in which it is present. The coding sequence can also be synthetic, either wholly or partly, based upon an APP gene or protein sequence. The APP gene which encodes the Aβ peptide has been cloned in a number of different species, including mouse: Yamada et al., 1987, *Biochem. Biophys. Res. Commun.* **149**, 665-671; rat: Shivers et al., 1988, *EMBO J.* 1365-1370; human: Kang et al., 1987, *Nature* **325**, 773-736; Ponte et al., 1988, *Nature* **331**, 525-527; Tanzi et al., 1988, *Nature* **331**, 527-530; Kitaguchi et al., 1988, *Nature* **331**, 530-533. Other APP genes can be identified and cloned routinely, e.g., using nucleic acid hybridization, antibody expression, or polymerase chain reaction (PCR), e.g., with conserved sequences. The source of the Aβ peptide can be the same species as the species in which it is to be expressed or it can be different. In a preferred example, the source of the Aβ peptide is mouse when it is to be expressed in a mouse.

A heterologous promoter sequence can be operably linked to a DNA sequence coding for an Aβ peptide. By "operably linked" it is meant that the heterologous promoter is joined to the Aβ peptide coding sequence in a manner to permit the expression of that sequence to be

controlled and production of the encoded Aβ peptide. The peptide can accumulate intracellularly or extracellularly. The heterologous promoter sequence is preferably a neuron-specific promoter sequence, i.e., a promoter sequence which is active in neuronal cells, preferably, the promoter sequence is more active in neuronal cells than other cell types. Examples of neuron-specific promoter sequences are neuron-enolase, Purkinje-cell protein, dystrophin, neurofilament, preferably, a neurofilament-light (NF-L) gene promoter, more preferably, a mouse NF-L gene promoter (30). Neuronal specificity can be increased, e.g., by including introns which are part of the gene's coding sequence, such as by including the first intron of the neurofilament gene. The heterologous promoter sequence can also be, e.g., amplifiable, inducible, developmentally-regulated, etc., alone or in combination with a neuron-specific promoter. Thus, the heterologous promoter can be a hybrid containing elements of several promoters, including the APP gene promoter itself.

A recombinant nucleic acid molecule according to the present invention is a non-naturally occurring nucleic acid molecule. Such a nucleic acid contains a unit of a promoter operably linked to a sequence which codes substantially only for Aβ peptides. It can be used in various ways, e.g., as a research tool or probe, e.g., or to effect expression of an Aβ peptide in a cell or whole mammal into which it is introduced. For expression purposes, such a recombinant DNA can also comprise a ribosome-binding site, translation initiation sequences, a Kozak consensus sequence, a coding sequence for APP, or a fragment thereof, termination sequences, polyadenylation sequences, i.e., those nucleic acid sequences useful to achieve expression of an Aβ peptide. A recombinant nucleic acid can further comprise enhancer sequences which modulate gene expression, intron sequences, and 5' and 3' flanking nucleotide sequences of

an APP gene or another desired gene. A nucleic acid sequence according to the present invention can be modified to improve expression of the nucleic acid sequence coding for Aβ, e.g., by the addition of enhancer sequences or by altering nucleotide sequence information to eliminate mRNA secondary structure which reduces or interferes with translation. See, e.g., *Methods in Enzymology*, Volume 185, Academic Press, 1990, especially Chapters 38-44. By "transgene" is meant a recombinant nucleic acid containing information to express an Aβ peptide in an animal into which it is introduced, e.g., promoter, a ribosome binding sequence, a Kozak sequence, i.e., CCPuCCAUGG or CCA/TCCA (see Kozak, Nucl. Acid. Res., 12, 857-872, 1984), translation initiation sequences, enhancer, intron, and/or polyadenylation sequence. Such a transgene can contain more than one coding sequence. These sequences used to effect expression are generally known in the art and discussed above.

The present invention also involves a transgenic mammal that contains in some or all of its cells a recombinant DNA, or transgene, comprising a promoter operably linked to a DNA sequence coding substantially for Aβ peptides. In an aspect of the invention, a heterologous promoter and Aβ peptide are obtained from the same species of mammal into which they are to be introduced by transformation; preferably, this species is a mouse.

The creation of the transgenic mammal can be accomplished routinely. A transgenic mammal can be made by, e.g., directly injecting a transgene into an embryo, using a retrovirus carrying the recombinant nucleic acid, or employing embryonic stem cell methodology. See, e.g., U.S. Patent Nos. 4,736,866; 4,873,191; 4,873,316; 5,082,779; 5,304,489; 5,174,986; 5,175,384; 5,175,385; and 5,221,778. To accomplish embryonic transformation, a recombinant DNA comprising the desired Aβ sequence is

introduced by microinjection into a pronucleus of fertilized one-cell embryo. See, e.g., Gordon et al., *Proc. Natl. Acad. Sci.*, 77:7380-7384 (1980); Palmiter et al., *Cell*, 41:343-345 (1985); Palmiter et al., *Ann. Rev. Genet.*, 20:465-499 (1986). Other methods can be used as well, with a primary purpose being to introduce the recombinant nucleic acid into the cells of the organism in a form in which it can be expressed. For example, the transgene can be injected into a fertilized mouse egg before fusion between the sperm and egg; thus, if integration into the genome DNA occurs, every cell of the embryo inherits the transgene.

The term "mammal" is used herein to include all mammals, except humans. It also includes an individual mammal in all stages of development, including embryonic and fetal stages. A transgenic "mammal" is any mammal containing one or more cells bearing genetic information received, directly or indirectly, by deliberate genetic manipulation at a subcellular level, such as by microinjection or infection with recombinant virus from another organism, other than from its parent. The term is not intended to encompass classical crossbreeding, but rather is meant to comprise mammals in which one or more cells receive a recombinant nucleic acid molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating nucleic acid. The term "germ cell line transgenic mammal" refers to a transgenic mammal in which the genetic information was introduced into a germ line cell, thereby conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information they, too, are transgenic mammals. A transgenic mammal can also be a chimera in which the genetic information, i.e., the recombinant DNA comprising an Aβ peptide is present in only some cells of the entire organism.

The genetic information may be foreign to the species of mammal to which the recipient belongs, foreign

only to the particular individual recipient, or genetic information already possessed by the recipient. The introduced gene may be differently expressed than the native gene, e.g., temporally, spatially, or quantitatively.

The recombinant nucleic acid or transgene can be introduced into any mammal, including a mouse (Hogan et al., 1986, in *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), pig (Hammer et al., *Nature*, 315:343-345, 1985), sheep (Hammer et al., *Nature*, 315:343-345, 1985), cattle, rat, or primate. See also, e.g., Church, 1987, *Trends in Biotech.* 5:13-19; Clark et al., 1987, *Trends in Biotech.* 5:20-24; and DePamphilis et al., 1988, *BioTechniques*, 6:662-680. In addition, e.g., custom transgenic rat and mouse production is commercially available.

In one aspect of the present invention, a recombinant DNA or transgene comprising a promoter operably linked to a DNA sequence encoding an Aβ peptide can be introduced into a mouse to form a transgenic mouse containing in its cells the recombinant DNA. The recombinant DNA can be present in either germ line or somatic cells, or both. The promoter, e.g., NF-L and Aβ coding sequence, is preferably from the mouse. Any known mouse can be used as a host for the transgene or recombinant DNA. For example, the host can be an in-bred strain, e.g., FVB/N, or it can be an out-crossed strain.

Another aspect of the invention is also the development of a transgenic mammal which reproduces itself, i.e., in which the germ-line of the transgenic mammal is transformed stably with the recombinant DNA according to the present invention, permitting its transmission to subsequent generations.

A further aspect of the invention is the expression of the Aβ peptide in the transgenic mammal. The occurrence of amyloid deposits comprising the Aβ peptide

is a characteristic feature of Alzheimer's disease (AD) and is a facet of the disease's neuropathology. Aβ deposition, or amyloidosis, is also observed in other diseases, including, e.g., Down's syndrome and heredity cerebral hemorrhage with amyloidosis Dutch type (HCHWA-D). Expression of the Aβ peptide in a transgenic mammal and its consequent phenotype can therefore be used a model for such diseases and pathologies, e.g., as an AD model. For example, active agents, e.g., synthetic, organic, inorganic, or nucleic acids based molecules, can be administered to a transgenic mammal according to the present invention to identify agents which either inhibit, prevent, and/or reduce the appearance of Aβ peptide in the brain, the AD pathology, neurodegeneration, apoptosis, cognitive deficits, and/or behavioral symptoms, etc. Thus, another aspect of the invention is to provide a method to assist in the advancement of the treatment and/or prevention of the aforementioned symptoms (e.g., neurodegeneration or apoptosis) caused by the APP gene, or a fragment thereof.

Such a mammal model can also be used to assay for agents, e.g., zinc, and factors, e.g., environmental, which exacerbate and/or accelerate the diseases. See, e.g., Bush et al., *Science* 265, 1464-1467, 1994.

Moreover, a mammal containing a transgene according to the present invention can be used in a method of screening a compound for its effect on a phenotype of an mammal, preferably a mouse, where the phenotype is conferred by the transgene. By "phenotype" is meant, e.g., a collection of morphological, physiological, biochemical, and behavioral traits possessed by a cell or organism that results from the interaction of the genotype and the environment. Such a phenotype can be behavioral, e.g., occurrence of seizures or cognitive performance, or it can be physiological and/or pathological, e.g., occurrence of neuronal cell degeneration, neuronal cell apoptosis, or accumulation of

Aβ peptide in the brain of the mammal. According to such a method of detection, a compound can be administered to an mammal containing a transgene and then the existence of an effect on the phenotype of the mammal can be determined. Observation can be accomplished by any means, depending on the specific phenotype which is being examined. For example, the ability of a test compound to suppress a behavioral phenotype can be detected by measuring the latter phenotype before and after administration of the test compound.

The invention also relates to a transgenic mammal that contains in its cells, preferably carried in the genome of it, a recombinant DNA comprising a promoter operably linked to a DNA sequence coding for an Aβ peptide and also having a phenotype characterized by the accumulation of the Aβ peptide in the brain, e.g., hippocampus, cerebral cortex, and amygdala, of the transgenic mammal, wherein the phenotype is conferred by the expression of the recombinant DNA.

The level of expression of the Aβ peptide can be any amount which can produce a phenotype in the mammal which phenotype can be distinguished from mammals which do not possess the transgene, i.e., a control mammal, e.g., an amount effective to produce neuronal cell degeneration and/or apoptosis and/or an amount effective to cause a behavioral and/or cognitive effect or dysfunction. A mammal containing the Aβ transgene can also be characterized by accumulation of the Aβ peptide in its brain. The accumulation can be in any quantity which is greater than that observed in mammals not containing the transgene. However, the phenotype conferred by the transgene can occur before or after accumulation can be detected. The expression and accumulation of Aβ peptide in the brain of the mammal can be measured conventionally, e.g., by immunoassay or nucleic acid hybridization, either *in situ* or from nucleic acid isolated from host tissues. In the examples which

follow, Aβ expression was virtually undetectable in controls on antibody-stained cryosections but clearly visible in mammals having the Aβ transgene. See, e.g., Figure 4.

The identification of agents which prevent and/or treat symptoms associated with expression of the Aβ peptide can be determined routinely. For example, an active agent can be administered to a transgenic mammal expressing a recombinant DNA according to the present invention and then its effect on a behavior or pathology, e.g., Aβ deposition in the brain, apoptosis, and/or neurodegeneration, can be determined. The agent can be administered acutely (e.g., once or twice) or chronically by any desired route, e.g., subcutaneously, intravenously, transdermally, or intracathically. The formulation of the agent is conventional, see, e.g., *Remington's Pharmaceutical Sciences*, Eighteenth Edition, Mack Publishing Company, 1990. In a test, e.g., an agent can be administered in different doses to separate groups of transgenic mammals to establish a dose-response curve to select an effective amount of the active agent. Such effective amount can be extrapolated to other mammals, including humans.

The transgenic mammal, preferably a mouse, according to the present invention therefore permits the testing of a wide variety of agents and therapies. In AD, for example, a number of different agents have been identified which affect the cognitive dysfunction associated with the diseases, e.g., cholinergic agents, such as muscarine agonists, acetylcholinesterase inhibitors, acetylcholine precursors, biogenic amines, nootropics, and angiotensin converting enzyme (ACE). In addition, agents which regulate Aβ expression, Aβ deposition, and physiological changes associated with Aβ expression and deposition can also be identified, e.g., calcium homeostasis, inflammation, neurofibrillary tangles. See, e.g., Pavia et al., *Annual Reports of*

Medicinal Chemistry, 25:2129, 1989; John et al., *Annual Reports of Medicinal Chemistry*, 28:197-203, 1993. Additionally, active agents which block apoptosis, e.g., free radical scavengers, such as glutathionines, can be administered. Such effects on AD can be assayed in either behavioral or physiological and/or histological studies.

For example, spatial learning and memory abilities in mice can be tested in a Morris water maze. See, e.g., Yamaguchi et al., *NeuroReport*, Vol. 2, 781-784 (1991). Additionally, other behavioral tests can be used, e.g., Swim Test, Morris et al., Learning and Motivation, 12, 239-260, 1981; Open-field test, Knardahl et al., *Behav. Neurol. Biol.* 27, 187-200, 1979; and tests and models used routinely, e.g., in mice, rats, and other rodents.

According to the present invention, differences in, e.g., levels of expression, cellular localization, and/or onset of expression of Aβ, can be used to model AD and other diseases associated with Aβ expression and the differing stages and progressions of the disease, e.g., cell degeneration, cell death, astrogliosis, and/or amyloidosis. As indicated in the examples which follow, quantities, temporal expression, and spatial localization of a transgenic Aβ peptide in a mouse, can be achieved. Having a range of Aβ peptide expression phenotypes can be useful to identify different therapies and drug treatments and also diagnostically to identify a disease's progression. For example, the specific treatments can depend on the region of the brain in which Aβ peptide is expressed, how much of it is expressed, and its temporal progression of expression. Thus, mammals having different Aβ peptide phenotypes can be used as models for determining therapies which are selective for different stages of the disease and for studying disease progression and intervention.

In another aspect of the invention, a recombinant DNA comprising the coding sequence for substantially Aβ

peptide and cells and/or mammals transformed with it can be used as a source of Aβ, i.e., as an Aβ factory. For example cells, e.g., see *ATCC Catalogue of Cell Lines and Hybridomas*, 7th edition, 1992, can be individually transformed with the recombinant DNA and selected for expression of Aβ. For methods on how to transform mammalian cells, see, e.g., *Methods in Enzymology*, Volume 185, 1990, especially, Chapters 34-44; *Molecular Cloning*, Sambrook et al., 1989, especially, Book 3, Chapter 16; EP 0 451 700. The Aβ peptide can be routinely isolated from the cell cultures, e.g., see Masters, C.L. et al., "Amyloid plaque core protein in Alzheimer disease and Down syndrome," *Proc. Natl. Acad. Sci. USA* 82:4245, 1985, and used, e.g., as a research tool, such as an antigen to raise antibodies or to determine the physical properties of Aβ for drug therapy design, analogously to how the commercially available amyloid β-protein fragments listed in the *Sigma Chemical Company Catalog*, page 1119, 1994, would be used. For such purposes, the Aβ peptide can also be isolated from cells or transgenic mammals according to conventional means, e.g., by removing tissues in which Aβ is expressed and purifying the peptide according to routine procedures. Cells expressing a recombinant DNA comprising an Aβ peptide can also be used as an *in vitro* model for studying Aβ expression and accumulation. For example, agents can be administered to cell cultures comprising Aβ expressing cells, and the effects of such agents can be studied. See, e.g., U.S. Patent No. 5,087,571. Thus, such cell cultures can be used as model systems, as well.

The present invention also relates to methods of preventing or treating Alzheimer's disease by interfering with the expression of intracellular Aβ peptide. Where previously it had been believed that secretion of the Aβ peptide from the cell and its consequent accretion into extracellular plaques was responsible for the characteristic cell death observed in AD patients, it has

now been shown that intracellular Aβ (Aβ while still in the cell) can trigger the physiological cascade leading to neuronal cell death, e.g., apoptosis, and other deleterious events associated with the disease. This indicates an earlier target for AD intervention than had heretofore been identified. For example, previous studies had concluded that the extracellular deposition of Aβ was a primary cause of the disease, leading investigators to suggest that interfering with plaque formation is sufficient to prevent or treat the disease. According to this aspect of the present the present invention, while extracellular Aβ plaque formation in the brain of afflicted patients can be deleterious to neuronal cells, it is not the only phenotype to be avoided to adequately treat the disease. In addition, expression of Aβ inside the cell, without plaque formation, can be a harmful event. Dissolution or prevention of the Aβ plaque after Aβ production may not be sufficient to achieve successful disease intervention, or optimal disease intervention.

Thus, an aspect of the present invention is, inhibiting or lowering the expression of intracellular Aβ peptide, e.g., by administering to a patient having Alzheimer's Disease, or a related disorder, an amount of a compound effective for such purpose. Typically, the amount of Aβ expression inhibited or lowered is less than the amount which can lead to the appearance of p53. As discussed in Example 9, the Aβ peptide initiates the cascade leading to apoptosis and cell death by causing the expression of p53. Thus, to ameliorate or prevent the symptoms associated with AD and related disorders, the expression of intracellular Aβ peptide can be reduced to levels at which the p53 gene is not activated, or the p53 gene is not expressed, or expression is insufficient to produce apoptosis. By "expression," it is generally meant any event which leads to the production and/or accumulation of intracellular Aβ or p53 peptide, e.g.,

transcription of the gene, translation of the corresponding mRNA, stability or perdurance of the gene product inside the cell.

Another aspect of the invention related to this finding is the treatment or prevention of AD by interfering with the expression of a p53 gene or its product. It has now been discovered that expression of intracellular Aβ can induce p53, leading to apoptosis and the extracellular release of Aβ peptide. By interfering with, e.g., blocking or inhibiting, the appearance of the p53 gene product inside the cell, AD can be treated or prevented.

Intervention with the expression of the APP or p53 gene can be accomplished conventionally, e.g., by interfering with gene transcription, gene translation, or the perdurance of gene product in the cell. For example, anti-sense polynucleotides or ribozyme inhibitors can be employed to inhibit translation of an encoding mRNA into a polypeptide. See, e.g., Yung WK, *Curr. Opin. Neurol.*, 7(6): 501-5 (1994). The methods for selecting a ribozyme or anti-sense nucleic acid compound can be determined based on the known sequence of the APP or p53 mRNA and tested *in vitro* for its effect on translation. [REFERENCE] The nucleic acid compound can be administered conventionally, e.g., by providing it as a pharmaceutical composition administered orally, intravenously, intrathecally, by stereotactic inoculation into the brain, etc., in an amount effective to inhibit gene translation. To increase the stability or other characteristics of the compound useful when administered to a patient, nucleotide derivatives can be substituted for the naturally-occurring bases.

A ribozyme or anti-sense compound can also introduced into the brain by a genetic vector. For example, a retroviral or adenovirus vector can be employed to transfer a gene encoding a ribozyme or anti-sense oligonucleotide into a patient. See, e.g., M.G.

Kaplitt et al., *Nat. Genet.* 8(2): 148-54 (1994); Horellou et al., *Neuroreport (ENGLAND)* 6(1): 49-53 (1994); G. Le Gal La Salle et al., *Science* 259: 988-90 (1993); S. Chatterjee et al. *Science* 258: 1485-8 (1992); M. Yamada et al., *Jpn. J. Cancer Res.* 83: 1244-7 (1992); Y. Takahara et al., *J. Virol.* 66: 3725-32 (1992). Herpes simplex virus 1 vectors can also be used to modify neuronal physiology *in vivo*, e.g., by the introduction of an anti-sense or ribozyme encoding gene, or other genetic elements which can be used to interfere with the expression of the APP or p53 gene. See, e.g., L. Soroceanu et al., *Proc. Natl. Acad. Sci.* 92(5); 1411-5 (1995); D.S. Latchman, *Mol. Biotechnol.* 2(2) 179-95 (1994); H.J. Federoff, et al., *Proc. Natl. Acad. Sci.* 89: 1636-40 (1992); A. I. Geller, *J. Neurosci. Methods* 36: 91-103 (1991). Compounds can also be administered by liposomes or by implanting cells expressing the desired compound into the brain. See, e.g., E.Y. Snyder et al., *Nature* 374(6520): 367-70 (1995).

Intervention with the expression of the APP or p53 gene can also be achieved by manipulating the cellular processes responsible for the processing of the gene product, such as cleavage of the precursor protein by regulating the corresponding enzymes or uncoupling the signal of the Aβ peptide to p53 expression. Such useful agents include, e.g., anti-oxidants, and can be administered routinely. Effective drug dosages can be determined from the animal model according to the present invention.

In a related facet, the detection of p53 gene activity, e.g., by detecting its mRNA, protein product, or antibodies to it, can be employed to diagnose or assess disease progression. The latter can be especially useful in the mouse model to assess the efficacy of different drugs in treating or preventing AD and related disorders. Thus, an effect of a compound, e.g., an anti-sense oligonucleotide, on the mouse AD phenotype can be

studied by measuring its ability to reduce or alter neuronal p53 levels. The present invention therefore relates to a method of identifying compounds to prevent or treat AD comprising administering a compound to a transgenic animal according to the present invention and measuring the amount of neuronal p53. The p53 can be quantified *in vivo* or *in vitro*, e.g., by sacrificing the animal, removing the brain and detecting p53 mRNA or protein on tissue sections or homogenates according to standard procedures, e.g., *in situ* hybridization, Northern analysis, PCR.

DNA, RNA, and other nucleic acid manipulations can be performed routinely, e.g., as described in Molecular Cloning, Sambrook et al., 1989.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

The entire disclosures of all applications, patents, and publications cited above and below are hereby incorporated by reference. The publication "The Alzheimer's Aβ Peptide Induces Neurodegeneration and Apoptotic Cell Death in Transgenic Mice", *Nature Genetics* 9, pp. 21-30 (1995), and the manuscript titled, "Extracellular Deposition of β-amyloid Upon p53-dependent Neuronal Cell Death in Transgenic Mouse," by Frank M. LaFerla, Catherine A. Kappel Hall, Lien Ngo, and Gilbert Jay, is also incorporated by reference and attached herewith.

BRIEF DESCRIPTION OF THE DRAWINGS

Various other objects, features, and attendant advantages of the present invention will be more fully appreciated as the same becomes better understood when considered in conjunction with the accompanying drawings, in which like reference characters designate the same or similar parts throughout the several views and wherein:

Figure 1 is a schematic representation of the *Hin*dIII-*Sac*I restriction fragment used to generate transgenic mice. The sequence encoding the Aβ peptide was generated by reverse transcriptase-polymerase chain reaction using rat brain RNA because it will yield a peptide identical to the mouse. The forward primer contained an ATG initiation codon flanked by the Kozak consensus sequence(61) (there are no other upstream ATG sites within this transcriptional construct) while the reverse primer contained an in-frame TAA termination codon. The 151-bp PCR fragment was cloned into the *Bam*HI and *Sac*II sites of the pBluescript-KSII vector (Stratagene), and was subsequently confirmed by sequence analysis. The SV40 polyadenylation-signal sequence was isolated by *Bam*HI and *Bcl*I digestion(62); *Sac*II linkers were ligated to this fragment which was then cloned downstream of the Aβ region. The mouse NF-L promoter(63) was cloned into the *Hin*dIII and *Bam*HI sites. The 2.2-kb *Hin*dIII-*Sac*I fragment was used for the generation of the transgenic mammals.

Figure 2 is a characterization of the Aβ mRNA in transgenic mice. Lanes 1 and 2 contain 10 μg of RNA from the brains of 4-week old non-transgenic and G2 transgenic mice, respectively. The higher molecular weight band in both lanes 1 and 2 correspond to the endogenous APP transcripts. In lane 2, the lower band (indicated by the arrow) is approximately 600 nucleotides (nt) in length and represents the recombinant DNA transcript. The position of the 28S and 18S ribosomal RNA are indicated by the arrowheads.

Figure 3 shows detection of the Aβ transgenic mRNA in the brain by *in situ* hybridization. Darkfield view of emulsion autoradiograms of coronal sections of brain from 6-month old non-transgenic (*a*) and transgenic (G2) (*b*) mice. An ^{35}S-labeled antisense oligonucleotide probe that recognized the Aβ and SV40 regions of the transgenic transcript was used for these *in situ* hybridizations. CA, Ammon's horn; CX, cerebral cortex; DG, dentate gyrus; HC, hippocampus.

Figure 4 shows detection of Aβ immunoreactivity in the brains of transgenic mice (G2). Brain cryosections from control and transgenic mammals are on the left and right sides, respectively. Brain regions represented include the hippocampus (*a,b*) and the cerebral neocortex (*c,d*). The arrows show apparent extracellular Aβ immunoreactivity. These sections were immunostained with the anti-Aβ antibody from Boehringer Mannheim. Original magnifications: a,b = 25X; c,d = 50X. CA, Ammon's horn; DG, dentate gyrus.

Figure 5 shows the incidence of death in the NF_L-Aβ transgenic mice. The solid and striped bars reflect the death rates for the control and transgenic mice, respectively. Significance values were calculated using Chi square (Maentel-Haenzel) analysis and are indicted above each time point.

Figure 6 shows alterations in neuronal cell morphology in the brains of transgenic mice. High magnification micrographs of hematoxylin-and-eosin stained sagittal sections from the neocortex (*a,b*), hippocampus (*c,d*), and thalamus (*e,f*) are shown. Control mice and transgenic littermates (10 months of age) are on the left and right sides, respectively. In *c* and *d*, the top tract of cells are from the dentate gyrus while the bottom are from the CA3 region. Original magnifications = 50X

Figure 7 shows biochemical and morphological detection of apoptotic cells in transgenic brains.

Cerebral cortex from a control mammal in which no TUNEL-positive cells are evident (*a*). Transgenic neocortex from the G2 (*b*) and J3 lines (*c*) stained with TUNEL showing extensive apoptosis. Serial sections from the J3 neocortex stained with either hematoxylin-and-eosin (*d*) or GFAP (*e*). A control hippocampus from a normal mammal in which no TUNEL-positive cells are evident (*f*). The hippocampus (*g*) and amygdala (*h*) from mammals of the G2 line with extensive TUNEL staining. Serial sections from the thalamus of a G2 transgenic mammal stained with TUNEL (*i*) or H & E (*j*), providing biochemical and morphological evidence of apoptosis. Arrows in *i* point to cells with perinuclear TUNEL staining, while in *j* they point to condensed, apoptotic cells; arrowheads point to fragmented nuclei (*i*, *j*). Original magnifications: a-e = 50X; f-j = 25X.

Figure 8 shows astrogliosis in the brains of transgenic mice. Low magnification micrographs of GFAP-immunostained sagittal brain sections showing the hippocampal and cortical regions of a 6-month old control mouse (*a*) and its transgenic littermate (*b*), original magnifications = 10X. Higher magnification micrographs of the neocortex from a control mouse (*c*) and transgenic mice from lines G2, J3, and J4 (*d-f*) are shown, original magnifications = 50X. CA, Ammon's horn; CX, cerebral cortex; DG, dentate gyrus; GL, glial limitans.

Figure 9 shows the amino acid sequences human (A), murine (B), and HCHWA-D (C) Aβ peptides. The amino acids are represented by the conventional one-letter symbols. A dash indicates that the amino acid is the same as the human sequence.

EXAMPLES

Example 1: DNA Construct Comprising an Aβ Peptide

A murine homolog of the human Aβ peptide was selected to interact with species-specific cellular factors to potentiate its toxicity. The murine and human Aβ peptides are highly conserved, and, like its human counterpart, the murine peptide possesses the ability to form fibrils *in vitro* and is as amyloidogenic as the human sequence(28,29). To restrict expression of the Aβ coding region to neuronal cells, 1.8-kb of 5′ flanking DNA from the mouse neurofilament-light (*NF-L*) gene was selected (Figure 1); the *NF-L* gene is transcriptionally active throughout adult life(30). To permit the translation of the chimeric transcript, a methionine initiation codon and a Kozak sequence was placed immediately upstream of the Aβ coding sequence. The polyadenylation sequence was derived from a BamHI/Bc1I digestion of SV40 DNA. There are two polyA signals on this fragment in opposite orientations; the early polyA signal of the T-Ag gene (i.e., 5′ Bc1I-BamHI 3′) was used. However, it has been found (*Mol. Cell. Biol.* 9, 4248-4258) that the polyA signal may be utilized if inserted in the late orientation, i.e., 5′ BamHI-BC1I 3′; thus, this fragment can be used, as well. For the promoter, the HindIII site is a naturally occurring site at the 5′-end of the promoter, and the BamHI was engineered (*J. Cell. Biol.* **108**, 579-593, 1989). After processing in the cell to remove the methionine, an authentic 42-amino acid Aβ peptide will be generated.

Example 2

The 2.2-kb NF_L-Aβ chimeric recombinant DNA was microinjected into single-cell FVB/N embryos(31-34). Founder mammals were backcrossed to the parental FVB/N (Taconic, 273 Hover Avenue, Germantown, NY 12526) strain to establish independent lines. The initial transgenic

mammals were identified by Southern blot hybridization. Tail DNA was extracted, digested with the restriction enzyme BamHI and subjected to electrophoresis on an agarose gel. The DNA was then transferred to nitrocellulose and probed with a DNA fragments which includes the 3' portion of the neurofilament promoter through the Aβ coding sequence.

To determine if expression of the recombinant DNA had been targeted to the brain, total RNA was prepared from an F_1 mammal from the various lines and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). In this semi-quantitative analysis, six of eight transgenic lines were identified as positive for RNA expression. To determine if the appropriate size transcript was generated and to determine the relative expression levels between the transgenic lines, Northern blot hybridization was performed (Figure 2). One line (G2) expressed the recombinant DNA to a significantly higher level than the other lines. The transgenic transcript is predicted to be approximately 500-600 nucleotides (nt) long, depending on the length of the polyA tract; this includes 111 nt of 5' non-coding sequence, 129 nt from the coding region, and another 175 nt of 3' non-coding sequence. The probe used for the Northern analysis, in addition to detecting the transgenic transcript, also detected the endogenous APP transcript, providing an internal reference to which the steady-state level of the recombinant DNA mRNA could be compared. Hybridization to a band of approximately 600 nt, the expected size of the transgenic transcript, is only seen in lane 2, which contains brain RNA from a transgenic mouse of the G2 line. As expected, the probe also detects a band of approximately 3.2-kb in length, the reported size of the endogenous APP transcripts(5-7), which is also present in the lane containing RNA from a nontransgenic brain. A comparison of the levels of the transgenic transcript to the endogenous transcript

clearly reveals a substantially higher steady-state level for the former (Figure 2). This difference is even more dramatic when one considers that the transgenic mRNA will exclusively encode the Aβ peptide while at best only a fraction of the molecules translated from the endogenous APP mRNA will yield Aβ. Based upon these RNA analyses, three independent expressing lines (G2, J3, and J4) were selected for further analysis.

Example 3

To precisely localize those areas of the brain that expressed the recombinant DNA, *in situ* hybridization analyses were performed.

In situ conditions: Paraffin sections of brain tissue on silane-coated slides were deparaffinized in xylene, rehydrated through an ethanol series, and treated with proteinase K (1 μg/ml) for 10 min. This was followed by treatment with 2 mg/ml glycine in PBS for 30 sec, a rinse in PBS, a postfixation in 4% paraformaldehyde in PBS for 5 min. Sections were then air dried and hybridized with ~ 4 x 10^6 cpm/50 μl/slide of tailed probe. Hybridization buffer consisted of 50% formamide, 0.6 M NaCl, 0.05 M Tris, 7.4, 0.004 M EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 10% dextran sulfate, 0.2% sodium heparin, and 0.1 M DTT. After overnight incubation at 37°C, slides were rinsed in 1 x SSC for 10 min. (twice), washed once for 10 sec. in water, dipped in 70% ethanol and air dried and then exposed to film.

Oligonucleotide probe for *in situ* hybridization:
Aβ/SV40 junctional probe,
5'GTGGTATGGCTGATTATGATCCGCGGTTATGCTATGACAACGCCACC3';
SV40 probe only,
5'TATGTTTCAGGTTCAGGGGGAGGTGTGGGAGG3'.

Coronal brain sections from the transgenic mammals and their nontransgenic littermates were hybridized with an oligonucleotide probe homologous to the junction between the Aβ and SV40 sequences present in the

recombinant DNA. Strong hybridization signal was observed in specific regions of the brains of transgenic mice (Figure 3b). Recombinant DNA expression was readily detected in the cerebral cortex and hippocampus, regions that are significantly compromised in AD. These results were also confirmed by hybridization to sagittal brain sections. Other regions of the transgenic brain, including the thalamus and hindbrain, expressed the recombinant DNA to a lesser degree. This same probe did not significantly hybridize to the brain section from a control mouse (Figure 3a), although a weak hybridization signal was detected in the hippocampal region which likely reflects background hybridization to endogenous App since a probe homologous to only the SV40 sequence at the 3' non-coding region of the recombinant DNA failed to hybridize. Moreover, the pattern of hybridization observed with the SV40 probe on sections from transgenic mammals was similar to that observed with the junctional probe (data not shown). While these studies clearly reveal that the recombinant DNA is expressed in neuronal cells, we cannot exclude the possibility that expression may also occur in other cell types.

To determine where the Aβ peptide was accumulating in the brain, cryosections from transgenic and age-matched control mice were immunostained with Aβ-specific antibodies. Antibodies used included Boehringer Mannheim Cat. No. 1 381 431; rabbit polyclonal antisera (472,473) prepared conventionally; and antibody 4G8, purchased from H. Wisniewski (SUNY Brooklyn, New York). Antibody to GFAP was purchased from Dakopatts (Cat. No. Z 334). No immunostaining was detected in either transgenic or control brain tissues when pre-immune serum was used (data not shown). Nontransgenic brain sections also failed to immunostain appreciably with any of the Aβ antibodies tested, whereas Aβ immunoreactivity was detected in the brains of transgenic mice.

Figure 4 compares the regional distribution of immunoreactivity for the Aβ peptide in the hippocampal and cortical regions of control and transgenic mice. While significant Aβ immunoreactivity was not evident in the control hippocampus (Figure 4*a*), immunostaining was detectable over cells of Ammon's horn (CA cells) as well as over cells of the dentate gyrus of a transgenic mammal (Figure 4*b*). Note that many cells in the dentate gyrus are immunoreactive while only clusters of cells in the CA regions are immunostained in this mammal, suggesting that antibody staining is specific for cells which accumulate the Aβ peptide. Occasional foci of immunoreactivity found in hippocampal areas of low cellular density may represent extracellular or dendritic deposits of Aβ (Figure 4*b*). Another region of the transgenic brain that showed extensive Aβ immunoreactivity was the cerebral cortex. Accumulation of Aβ was observed over neurons throughout the cortex (Figure 4*d*), while a similar region from a nontransgenic littermate did not show any immunoreactivity (Figure 4*c*). Other regions that showed Aβ immunoreactivity included the thalamus and hindbrain, and again, those regions in the control brain did not reveal any immunoreactivity (data not shown). Interestingly, in every transgenic section, some cells showed prominent immunostaining while other neighboring cells failed to stain. This suggests that it is unlikely for the antibody to be binding non-specifically to different brain regions. Moreover, expression is not widespread in all regions; in some areas, expression appears higher and involves a majority of cells, whereas only focal areas of cells in other regions appear immunoreactive. By and large, there is a good overlap between Aβ mRNA and peptide expression in the brains of the transgenic mammals.

Example 4: Behavioral phenotype, neuronal cell degeneration results from Aβ expression

Initial observations of F_1-generation transgenic mammals revealed several cases of unexplained premature death. To analyze the incidence of death, a population of 76 transgenic mice and 43 nontransgenic littermates were studied. Mortality rates were calculated based on the number of mice that died relative to the number surviving within the specified time interval. These values are illustrated in Figure 5. A statistically significant difference between the rates of mortality for the transgenic and nontransgenic mice was apparent by six months of age ($P<0.05$). This difference became even more dramatic at 12 months when over 50% of the transgenic mice had died ($P<0.0001$). Since control FVB mice usually live to approximately 24 months of age, expression of the recombinant DNA may progressively impair vital neurological functions and result in premature mortality. While AD patients may live for many years with this affliction, the progressive impairment in the brain eventually leads to death in mid- to late-adult life(35,36).

Behavioral evidence of neurological abnormalities, in the form of seizures, were observed in several of the transgenic mice. These events generally persisted for 10-30 seconds during which the mice would extend all four limbs, open their mouths, flex their tails so that they became rigid, and shake violently. Following the episodes, the mice appeared to experience a brief period of lethargy. Given the brief nature of these seizures, it is likely that a larger proportion of the transgenic mice may, in fact, manifest this behavior. Furthermore, seizure activity may be triggered by disruption of specific neural pathways. Since not every mouse is affected to the same degree in each brain region, one may not expect all of the mammals to be equally involved. Seizures were never seen in any of the control littermates. Notably, the occurrence of seizures has

also been observed in AD patients, where it appears to be a frequent feature in later stages of the disease(37-39).

Transgenic mammals that died prematurely were necropsied to determine the cause of death. Gross anatomic as well as histological examination of visceral organs failed to reveal any pathological changes that would account for their deaths. However, in virtually every mammal, certain regions of the brain contained morphologically altered cells. To confirm this observation, age-matched control and transgenic mammals were anesthetized, transcardially perfused, and immediately processed for histological analysis. In the transgenic mice analyzed, sharply demarcated zones of unstained perinuclear cytoplasm of the neuronal cells were observed in different brain regions, including the cerebral cortex, hippocampus, thalamus, and occasionally the hindbrain. In those regions, affected cells were frequently found in the immediate vicinity of unaffected cells, suggesting that the observed changes were not a consequence of improper fixation. Another indication that the distinct perinuclear zone seen in the neurons of the transgenic mice were not due to tissue shrinkage was the appearance of the perivascular Virchow-Robin spaces, which were equally visible in both transgenic and control mice subjected to fixation by vascular perfusion.

Figure 6a shows the neocortex from a perfused nontransgenic mouse where the perikarya appear normal. In contrast, many cells in the transgenic cortex appear to be morphologically altered, as indicated by perinuclear zones of unstained cytoplasm. These altered cells were typically confined to focal regions within the cerebral cortex, but can be quite extensive (Figure 6b). The hippocampus was another region that frequently contained morphologically altered cells. While the overall structure of the hippocampus was not changed, note that neuronal cells with unstained cytoplasm are readily recognizable within the CA3 region and within the

dentate gyrus (Figure 6*d*). In contrast, these changes are not present in the control mammal (Figure 6*c*).

Degenerative neuronal cells were also observed in the thalamus (Figure 6*f*), and to a lesser extent the hindbrain (data not shown). Similar regions from negative littermates, processed in parallel, did not contain any altered cells (Figure 6*e*). Interestingly, these altered cells were not observed in the cerebellum, though it is possible that the degeneration of cerebellar cells does not proceed with the same morphological features. Although there is a good regional correlation between the accumulation of Aβ and the presence of altered cells, the limited morphological resolution of the cryosections did not allow us to unequivocally determine whether accumulation of the peptide always coincided with altered morphology. It remains possible that expression of Aβ in one cell may induce degeneration in a neighboring cell. In the transgenic mice, morphologically altered neuronal cells were observed in 18 of 19 mammals that died unexpectedly, as well as in 7 of 8 mammals that were euthanized by perfusion during the course of the study. Therefore, intracellular accumulation of the Aβ peptide may be deleterious to certain neurons or may render them more susceptible to other insults or challenges.

Example 5: Neurodegeneration is <u>followed by apoptotic cell death</u>

Examination of histological sections revealed that the nuclear chromatin in some cells was condensed and abutting the nuclear membrane, a distinguishing morphological feature of apoptosis(40). A biochemical hallmark of cells undergoing apoptosis is the internucleosomal cleavage of DNA(40) which is frequently visualized by gel electrophoresis. Unfortunately, this method is an averaging assay and is insensitive compared to assays like TUNEL (terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labelling) that

allow detection of dying cells *in situ*, before the DNA is extensively fragmented(41). In addition, TUNEL facilitates the determination of the actual number of cells undergoing apoptosis, allows a comparison of the extent of involvement between affected regions, and even allows the detection of a single, involved cell among a background of normal cells.

A survey of 28 transgenic mammals representing 3 independent lines revealed the presence of apoptotic cells in the brains of 14 of these mammals. Although apoptotic areas could be quite extensive in some mammals, they were generally focal; hence, it is possible that even more mammals contained apoptotic cells, since TUNEL was performed on random brain sections from each mammal. Moreover, these foci of apoptotic cells were more frequently observed in certain brain regions, implying that some areas were more susceptible than others. The cerebral cortex was one of the most frequently involved regions, and examples from representative mammals of the G2 and J3 lines are shown (Figures 7*b*, *c*). Note that TUNEL-positive cells are scattered throughout the most peripheral layers of the cortex; however, apoptotic cells were occasionally observed in the deeper cortical layers. We also observed that some of the nuclei were stained to different extents by TUNEL; as the nucleus was not condensed in some of these cells, it suggests that they may be at different stages of the apoptotic process (Figure 7*c*). In contrast, TUNEL-positive cells were not observed in the negative littermates (Figure 7*a*). In addition, cells with perinuclear zones of unstained cytoplasm (Figure 7*d*) were often found in close proximity to intensely stained TUNEL cells (Figure 7*c*), implying that the degenerating cells will eventually die by apoptosis.

The hippocampus was another region that frequently contained striking TUNEL staining in the transgenic mammals. While in some mammals, tracts of cells within

the CA1 and CA3 regions contained prominent TUNEL staining (Figure 7*g*), in others, cells of the CA2 region and dentate gyrus may also be labelled by TUNEL. No TUNEL-positive cells were observed in the control hippocampus (Figure 7*f*). Another prominent region that contained apoptotic cells was the amygdala (Figure 7*h*). In the amygdala, as was the case for the cerebral cortex and hippocampus, apoptotic cells were found in close apposition to unstained cells, demonstrating the specificity of TUNEL. Interestingly, these three brain regions that most frequently contained apoptotic cells are among the most compromised regions in AD brains(2).

Morphologically, under light microscopy, cells undergoing apoptosis are characterized by cell shrinkage and the condensation of the nuclear chromatin initially into dense patches around the nuclear membrane, then into crescent-shaped masses, and finally into dense spheres(42). Although TUNEL allows for earlier detection of apoptotic cells than morphological criteria alone(41), apoptotic cells were nevertheless recognizable in histological preparations. As expected, microscopic examination of brain sections indicated that areas containing TUNEL-positive cells also contained cells with distinct morphological features of apoptosis, including the presence of compacted and fragmented nuclei (cf. Figures 7*i*, *j*). Therefore, apoptotic cells could be clearly distinguished in the brains of the transgenic mice by both biochemical and morphological criteria.

Interestingly, brain regions in which extensive apoptosis was observed, as indicated by TUNEL and the presence of fragmented nuclei, also appeared hypocellular and we observed that the surrounding neuropil also assumed a secondary change, appearing somewhat "spongy." These islands of "spongy" tissue were found within regions of extensive neuronal cell degeneration (cf. Figures 7*i*, *j*). In human AD, a similar change in the underlying brain tissue has also been described(43). As

apoptosis has been speculated to be involved in neurodegenerative disorders(44), these results are important not only because they provide direct evidence for cell loss as a consequence of Aβ expression *in vivo*, but also suggest that the underlying mechanism responsible for cell death in AD involves apoptosis. Interestingly, Aβ has also been shown to induce apoptosis in cultured neurons(45).

Example 6: Reactive gliosis accompanies neurodegeneration and apoptosis

Since Aβ expression was inducing neurodegeneration and apoptosis in the brains of the transgenic mice, this suggests that there must be functional impairments as well. A prominent response to many types of injuries or insults to the CNS is the activation of astrocytes, a process referred to as gliosis(46); this reactive process is also frequently observed in AD brains(47-49). Consequently, brain tissues from transgenic and age-matched control mice were immunostained for glial fibrillary acidic protein (GFAP), an astrocyte-specific intermediate filament(50).

In the control brain, GFAP immunoreactivity was principally located in the hippocampal region and the glial limitans layer of the cerebral cortex (Figure 8*a*), in agreement with the reported distribution of GFAP-expressing cells(51). In contrast, brain sections from transgenic mice show greatly increased immunostaining (Figure 8*b*). Furthermore, in the transgenic brains from each independent line, the extent of GFAP immunoreactivity varied; in some mammals, gliosis was focal, but in others it was more widespread and extensive. For instance, in the G2 line, extensive gliosis was present throughout the cortical layers (Figure 8*d*), a region in which GFAP-positive astrocytes are not evident in normal mammals (Figures 8*c*). In the J3 and J4 lines, the gliotic areas were less widespread and may be more restricted to either the superficial or deeper cortical

layers (Figure 8e, f, respectively). The difference in extent of gliosis may be due to the lower level of recombinant DNA expression in the J3 and J4 lines. In addition, the astrocytes in the transgenic brains were enlarged, with thicker and increased numbers of processes, all features of reactive astrocytes(46). These findings strongly suggest that astrogliosis has been a response in conjunction with the neuronal changes observed in the transgenic brains. Astrogliosis was observed in the brains of at least 15 of 20 transgenic mammals examined that were over six months of age.

Reactive astrocytes are frequently found in areas that contained degenerating cells, either with or without cells progressing to apoptosis (cf. Figures 7c, e). This was true not only for the cerebral cortex but for other regions including the amygdala and hippocampus (data not shown). However, regions that appeared hypocellular and spongiotic were often not gliotic, suggesting that once cells have died, astrocytes were no longer present.

The development of astrogliosis in the transgenic mice provides further evidence for neurological abnormalities. Astrocytes play an important role in the CNS by responding to injury. Clearly, if the NF_L-Aβ recombinant DNA were provoking neuronal degeneration or damage, the development of gliosis would be a consistent and expected response. In the AD brain, pronounced and widespread gliosis has also been shown to be a striking and frequent feature(47-49). Curiously, in some transgenic mice, the presence of gliosis was noted in regions in which only minor neuronal changes were observed. This suggests that while the changes in neuronal morphology are a common feature in these mice, they are not the only form of neuronal damage that will elicit the involvement of astrocytes. Moreover, it is conceivable that astrocytes may play a contributory role in the degenerative process. This is supported by the

presence of focal areas of reactive astrocytes in the AD brain which are devoid of senile plaques(51).

Example 7

Recombinant DNA expression was detected in a variety of tissues including heart, intestine, kidney, liver, muscle, and spleen (data not shown), an observation that has been reported by others using the same NF-L promoter to drive expression of an heterologous gene in transgenic mammals(53). Since single cell analysis was not performed, it remains possible that expression was still limited to neurons in all of these tissues. Although recombinant DNA RNA expression was detected in peripheral tissues, histopathological changes were observed only in the brain. Interestingly, Aβ deposits can be detected in AD patients outside the CNS in tissues such as skin and intestine, albeit with only subclinical consequences(54). These observations suggest that the brain may be more vulnerable to the toxic effects of the Aβ peptide both in humans and in mice.

Even within the brain there appears to be more susceptible areas; despite expression of the recombinant DNA throughout the brain, neurodegeneration and apoptosis were more frequently observed in the cerebral cortex, hippocampus and amygdala, less extensively in the hindbrain and virtually undetectable in the cerebellum. It should be pointed out that the Aβ peptide by itself may not suffice to induce neurotoxicity but that it may act in concert with other factors. Accumulation of Aβ within neuronal cells of the brain may render those cells more susceptible to different insults or challenges. This may contribute to the variable time of disease onset. While we have not assayed for a negative control peptide, our observation of a consistent phenotype in multiple, independent lines of transgenic mice leaves no doubt that the neurodegenerative phenotype is a consequence of recombinant DNA expression; since the recombinant DNA is programmed to produce only Aβ, the

phenotype that results in these mice can only be attributed to Aβ or a subspecies of it. Furthermore, it is worth noting that other transgenic constructs with the NF-L promoter driving expression of heterologous genes did not induce any brain pathology(53,55-57), indicating that changes observed in the NF_L-Aβ transgenic mice are a consequence of Aβ expression.

Despite the presence of profound changes in our transgenic brains, other diagnostic features of AD such as neurofibrillary tangles and senile plaques as distinguishable by Bielschowsky's silver stain were not observed, although some apparent extracellular Aβ immunostaining was detected within the neuropil. However, we will be exploring our aging transgenic population for the presence of these structures, although tangles are not necessarily an invariant feature of AD[1]. With regard to the senile plaques, it is conceivable that mice may never develop them as their normal life span is not long enough to permit the formation of such structures. Additionally, it has not been discounted that a cofactor(s) required for the genesis of plaques is absent in mice. Therefore, the marked pathological changes in the brains of the NF_L-Aβ transgenic mice, in the absence of senile plaques, may suggest that their presence is a consequence or secondary feature of this disease, and not a direct cause of the neuronal degeneration. Furthermore, we have generated transgenic mice in which Aβ was targeted extracellularly by including the N-CAM signal sequence(53), however, these mice have not developed any pathology compared to the transgenic mice expressing Aβ intracellularly; these results suggest that intracellular expression of Aβ leads to pathological changes while extracellular expression does not. In addition, our results now allow us to address which feature of Aβ (i.e., its primary sequence, hydrophobicity, or size) contributes to the neurodegenerative phenotype.

Example 8

An agent is administered to five different groups of transgenic mice (n = 10) expressing the transgenic Aβ peptide, each group differing by two months in age. The behavior of each mouse is compared to control mice of the same age using the Morris water maze, and the phenotype is observed. After observing and detecting the behavior, each mouse is sacrificed and assayed for Aβ expression as described in the examples above. The experiment is repeated administering different dosages of agent.

Example 9

Using an antibody directed against the N-terminus of p53 (19), we confirmed by immunostaining of formalin-fixed brain sections that neurons in the cerebral cortex on control mice do not normally express p53. In contrast, cortical neurons from Aβ transgenic mice (n=10) showed extensive p53 immunostaining. However, not every neuron was found to express p53; the extent of involvement varied from subregion to subregion and from mouse to mouse, perhaps suggesting that the intrinsic ability to express intracellular Aβ was not sufficient. The addition of the synthetic peptide to which the antibody was derived completely blocked p53 immunostaining. It is interesting to note that while a majority of the detectable cortical neurons in this transgenic mouse showed predominantly nuclear p53 immunostaining, a significant subset showed both nuclear and cytoplasmic localization, and an occasional subset frequently arranged ass a cluster may exhibit only cytoplasmic accumulation. It is not clear whether the difference in subcellular location of p53 represents different functional states of the cells or whether it characterizes responses of different neuronal subtypes. Regardless, activation of p53 is a distinguishing feature of the Aβ transgenic mice and correlates well with recent

in vitro studies which suggest that Aβ induces oxidative injury (65-67), an established inducer of p53 (68).

Does Activation of p53 correlate with Aβ accumulation? To determine whether sustained expression of Aβ is required to induce p53, we selected for analysis an Aβ transgenic mouse with a well-circumscribed area of apoptotic cells juxtaposed to an apparently normal area. Cell death by apoptosis, in contract to necrosis, is characterized by the presence of excessive DNA 3'-hydroxyl ends which can be detected using the terminal transferase (TdT)-mediated dUTP-biotin-nick-end labeling (TUNEL) method (69). From the relative signal strengths of the labeled nuclei, cells in early stages of the apoptotic pathway with marginal morphological changes can be distinguished from cells in advanced stages that have condensed or even fragmented nuclei.

Serial sections of the neocortex from an Aβ transgenic mouse were subjected to hematoxylin-and-eosin (H&E), TUNEL, anti-Aβ and anti-p53 staining. The cortical neurons were morphologically normal-appearing and exhibited an open nuclear morphology. As expected, these cells were TUNEL$^-$ because they had no Aβ accumulation and no p53 activation. In contrast, the cortical neurons located at the bottom left revealed a distinctively basophilic cytoplasm and appeared degenerative. They were weakly TUNEL$^+$ and showed abundance of both cytoplasmic Aβ and nuclear p53 immunoreactivity. The cortical neurons at the top right showed highly condensed or fragmented nuclei and were strongly TUNEL$^+$; many of these cells also expressed Aβ and p53. Since clusters of neurons invariably undergo apoptosis coordinately, the regional analysis of gene expression may be taken to represent responses in individual cells. This finding demonstrates that intracellular accumulation of Aβ correlated perfectly with the activation of p53.

Does p53 activation precede cell death? Since analysis of the cerebral cortex hinted to the expression of p53 occurring prior to the detection of overt cell death, we analyzed another region of the brain to determine whether even more convincing evidence may be obtained. Serial sections of hippocampus from another Aβ transgenic mouse were subjected to H&E, TUNEL and anti-p53 staining. The hippocampal neurons were observed to undergo progressive morphological changes along the horn of Ammon. IN the CA2 subregion, the cells appeared fairly normal, showed no TUNEL labeling but already had abundance of nuclear p53 immunoreactivity. In contrast to control mice, one can conclude that p53 had been activated although the cells had not undergone apoptosis. At the junction between the CA2 and CA3 subregions, the cells had acquired significant morphological changes and had a markedly altered nuclear-to-cytoplasmic ratio. These cells became weakly TUNEL' but maintained a high level of nuclear p53 immunoreactivity, suggesting that p53 is required for these cells to enter the apoptotic pathway. Toward the CA3 subregion, the cells had condensed chromatin structures and nuclei, andd were highly TUNEL' but were no longer p53 immunoreactive. This finding is consistent with the activation of p53 before the onset of apoptosis and its disappearance late in the process.

Is there extracellular deposition of Aβ in the brain? Using an anti-Aβ antiserum (4G8) that is well-characterized and widely-used(70), as well as with antisera that we ourselves have generated, we have detected extracellular Aβ immunostaining in many of the transgenic mice analyzed. As predicted, these deposits were found in different brain regions, particularly within the neocortex, hippocampus and thalamus. The locations of the three general areas can be deduced from parallel brain sections treated with either H&E or Bielschowsky's silver stain. The top left region

includes the CA1 subregion of the hippocampus and the overlying white matter (Splenium corporis callosi). The top right region lies within the CA3 subregion of the hippocampus and the overlying white matter (Truncus corporis callosi). The bottom left region is located within the thalamus (possibly Nucleus ventralis thalami, pars basalis).

A high magnification view of the anti-Aβ immunostaining in all three regions shows that the extracellular deposits resemble diffuse plaques. Interestingly, these deposits could also be detected with facility using a silver stain when found not overlying nerve fibers. Not only does this imply significant amounts of the Aβ protein being deposited in the extracellular space, a high magnification view also showed that these deposits assume the appearance of ice crystals and may represent fibrillar aggregates.

Could the extracellular deposits come from dying cells? Recognizing that a dying neuron has an abundance of cytoplasmic Aβ, it is important to determine whether Aβ could be released into the immediate extracellular space upon the death of the cell. This is particularly relevant since the manner by which Aβ gets out of the cell in AD has not been defined. Analysis of the hippocampal CA1 and CA3 cells, in the vicinity of the Aβ deposits, showed evidence of significant cell degeneration. For example, there is recognizable paucity of neurons within the CA3 subregion and some of the remaining cells have nuclei that were poorly stained by hematoxylin and nucleoli that could not be identified by Bielschowsky's silver stain.

More significantly, TUNEL· cells were found to perfectly overlap regions with extracellular Aβ immunostaining, confirming the association of cell death with extracellular deposition. Further evidence for cellular injury was obtained from H&E staining of regions around CA1 and CA3, which are hypocellular. The presence

of focal regions of basophilia, detected by hematoxylin which stains nuclear chromatin, an overlying Aβ immunoreactive areas, suggests that these deposits are released subsequent to cell death. At high magnification, an occasional cell ghost may be detected together with disorganization of the surrounding neuropil in an area marked by distinct basophilia.

Does extracellular Aβ deposition have functional consequences? Due to the localized nature of the Aβ deposits and its insoluble appearance, it is possible that the underlying neurons as mere bystanders may become functionally impaired. Guided by the fact that senile plaques in AD frequently involved not only extracellular Aβ deposits but also dystrophic neurites and reactive astrocytes (71), we analyzed for alterations in synaptophysin andd glial fibrillary acidic protein (GFAP) immunostaining. It was interesting to find that regions of the brain which normally have an abundance of astrocytes, including regions of the corpus callosum overlying the CA1 and CA3 subregions of the hippocampus, showed detectable gliosis. The involved astrocytes became morphologically activated which gave the areas a denser appearance upon GRAP immunostaining.

Underlying neuritic involved in these extracellular deposits was suggested by a marked increase in synaptophysin immunostaining within regions which normally have a basal level of immunoreactivity, such as areas immediately adjacent to the CA1 and CA3 neurons. Since the increased immunostaining precisely overlapped the Aβ deposits, it may represent the entrapment of underlying neurites. Interestingly, the presence of synaptophysin immunoreactivity has also been demonstrated in AD plaques (72). Further evidence for actual impairment of neurites was obtain from the use of Bielschowsky's stain which highlighted the fine structure of the neurites extending from the hippocampal neurons. Unlike those not underlying the deposits, which have fine

hair-like structures, many of those found underlying the Aβ deposits appears blunted with knob-like structures at their ends. This observation may very well suggest impairment of bystanding neurons and, possibly result in a second level of cell death.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

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WE CLAIM:

1. A mouse comprising cells which contain a transgene, said transgene comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for a mouse Aβ peptide.

2. A mouse according to claim 1, wherein the Aβ peptide has a sequence of Figure 9B.

3. A mouse according to claim 1, wherein the promoter is from a mouse.

4. A mouse according to claim 1, wherein the promoter is neuron-specific.

5. A mouse according to claim 4, wherein the neuron-specific promoter is a mouse NF-L promoter.

6. A mouse according to claim 2, wherein the promoter is a mouse NF-L promoter.

7. A mouse according to claim 1, wherein the Aβ peptide is expressed in the brain of said mouse in an amount effective to produce neuronal cell degeneration and/or apoptosis.

8. A mouse according to claim 1, wherein the Aβ peptide is expressed in the brain of said mouse in an amount effective to cause a behavioral or cognitive dysfunction, wherein the dysfunction is conferred by said transgene.

9. A mouse according to claim 1, wherein said cells are somatic and/or germ.

10. A mouse cell containing a transgene, said transgene comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for a mouse Aβ peptide.

11. A mammal comprising cells which contain a transgene, said transgene comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for an Aβ peptide, wherein the Aβ peptide is obtained from the same species of mammal which contain said transgene.

12. A transgene comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for a mouse Aβ peptide.

13. A transgene according to claim 12, wherein the Aβ peptide has a sequence of Figure 9B.

14. A transgene according to claim 12, wherein the promoter is from a mouse.

15. A transgene according to claim 12, wherein the promoter is neuron-specific.

16. A transgene according to claim 15, wherein the neuron-specific promoter is an NF-L promoter.

17. A transgene according to claim 15, wherein the neuron-specific promoter is a mouse NF-L promoter.

18. A transgene according to claim 12, further comprising a translation initiation sequence CCPuCCAUGG.

19. A transgene comprising a heterologous promoter operably linked to a nucleic acid sequence coding for a mouse Aβ peptide, wherein said sequence is not the complete normal mouse APP gene.

20. A recombinant nucleic acid comprising a promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for a mouse Aβ peptide.

21. A recombinant nucleic acid according to claim 19, wherein the Aβ peptide has a sequence of Figure 9B.

22. A recombinant nucleic acid according to claim 19, wherein the promoter is a mouse NF-L promoter.

23. A method of screening a compound for an effect on a phenotype mediated by expression of a transgenic Aβ peptide in the brain of a mammal, comprising:

administering said compound to a mouse expressing said transgenic Aβ peptide, and

observing whether an effect on said phenotype results.

24. A method for producing a transgenic mammal having a phenotype mediated by expression of a transgenic Aβ peptide in the brain of said mammal, wherein the Aβ peptide is expressed in the brain of said mouse in an amount effective to produce neuronal cell degeneration and/or apoptosis and/or in an amount effective to cause a behavioral or cognitive dysfunction, said method comprising:

(a) introducing a transgene into a zygote of a mouse, said transgene nucleic acid comprising a promoter operably linked to a nucleic acid sequence coding for said mammal Aβ peptide,

(b) transplanting said zygote into a pseudopregnant mammal,

(c) allowing said zygote to develop to term, and

(d) identifying at least one transgenic offspring containing said transgene.

25. A method for producing a transgenic mouse having a phenotype mediated by expression of a transgenic peptide in the brain of said mammal, wherein the Aβ peptide is expressed in the brain of said mouse in an amount effective to produce neuronal cell degeneration and/or apoptosis and/or in an amount effective to cause a behavioral or cognitive dysfunction, said method comprising:

(a) introducing a recombinant DNA into an embryo of a mouse, said recombinant DNA comprising a promoter operably linked to a DNA sequence coding for said mammal Aβ peptide,

(b) transplanting said embryo into a pseudopregnant mammal,

(c) allowing said embryo to develop to term,

(d) identifying at least one transgenic offspring containing said recombinant DNA, and

(e) breeding said offspring to form a transgenic line of mammal having said phenotype.

26. The method of claim 25, wherein said introducing of said transgene into said embryo is by introducing an embryonic stem cell containing said transgene into said embryo.

27. The method of claim 25, wherein said introducing of said transgene into said embryo is by infecting said embryo with a retrovirus containing said transgene.

AMENDED CLAIMS
[received by the International Bureau on 9 April 1996(09.04.96); original claims 11 and 23-25 amended; new claim 28 added; remaining claims unchanged (3 pages)]

9. A mouse according to claim 1, wherein said cells are somatic and/or germ.

10. A mouse cell containing a transgene, said transgene comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for a mouse Aβ peptide.

11. A non-human mammal comprising cells which contain a transgene, said transgene comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for an Aβ peptide, wherein the Aβ peptide is obtained from the same species of mammal which contain said transgene.

12. A transgene comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for a mouse Aβ peptide.

13. A transgene according to claim 12, wherein the Aβ peptide has a sequence of Figure 9B.

14. A transgene according to claim 12, wherein the promoter is from a mouse.

15. A transgene according to claim 12, wherein the promoter is neuron-specific.

16. A transgene according to claim 15, wherein the neuron-specific promoter is an NF-L promoter.

17. A transgene according to claim 15, wherein the neuron-specific promoter is a mouse NF-L promoter.

18. A transgene according to claim 12, further comprising a translation initiation sequence CCPuCCAUGG.

19. A transgene comprising a heterologous promoter operably linked to a nucleic acid sequence coding for a mouse Aβ peptide, wherein said sequence is not the complete normal mouse APP gene.

20. A recombinant nucleic acid comprising a promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for a mouse Aβ peptide.

21. A recombinant nucleic acid according to claim 19, wherein the Aβ peptide has a sequence of Figure 9B.

22. A recombinant nucleic acid according to claim 19, wherein the promoter is a mouse NF-L promoter.

23. A method of screening a compound for an effect on a phenotype mediated by expression of a transgenic Aβ peptide in the brain of a non-human mammal, comprising:

administering said compound to said mammal expressing said transgenic Aβ peptide, and

observing whether an effect on said phenotype results.

24. A method for producing a non-human transgenic mammal having a phenotype mediated by expression of a transgenic Aβ peptide in the brain of said mammal, wherein the Aβ peptide is expressed in the brain of said mammal in an amount effective to produce neuronal cell degeneration and/or apoptosis and/or in an amount effective to cause a behavioral or cognitive dysfunction, said method comprising:

(a) introducing a transgene into a zygote of a mammal, said transgene nucleic acid comprising a promoter operably linked to a nucleic acid sequence coding for said mammal Aβ peptide,

(b) transplanting said zygote into a pseudopregnant mammal,

(c) allowing said zygote to develop to term, and

(d) identifying at least one transgenic offspring containing said transgene.

25. A method for producing a transgenic mouse having a phenotype mediated by expression of a transgenic peptide in the brain of said mammal, wherein the Aβ peptide is expressed in the brain of said mouse in an amount effective to product neuronal cell degeneration and/or apoptosis and/or in an amount effective to cause a behavioral or cognitive dysfunction, said method comprising:

(a) introducing a recombinant DNA into an embryo of a mouse, said recombinant DNA comprising a promoter operably linked to a DNA sequence coding for said mammal Aβ peptide,

(b) transplanting said embryo into a pseudopregnant mammal,

(c) allowing said embryo to develop to term,

(d) identifying at least one transgenic offspring containing said recombinant DNA, and

(e) breeding said offspring to form a transgenic line of mice having said phenotype.

26. The method of claim 25, wherein said introducing of said transgene into said embryo is by introducing an embryonic stem cell containing said transgene into said embryo.

27. The method of claim 25, wherein said introducing of said transgene into said embryo is by infecting said embryo with a retrovirus containing said transgene.

28. A non-human mammal comprising cells which contain a transgene, said transgene introduced into a zygotic cell of said mammal or an ancestor of said mammal and comprising a heterologous promoter operably linked to a nucleic acid sequence consisting essentially of a coding sequence for an Aβ peptide, wherein the Aβ peptide is obtained from the same species of mammal which contain said transgene.

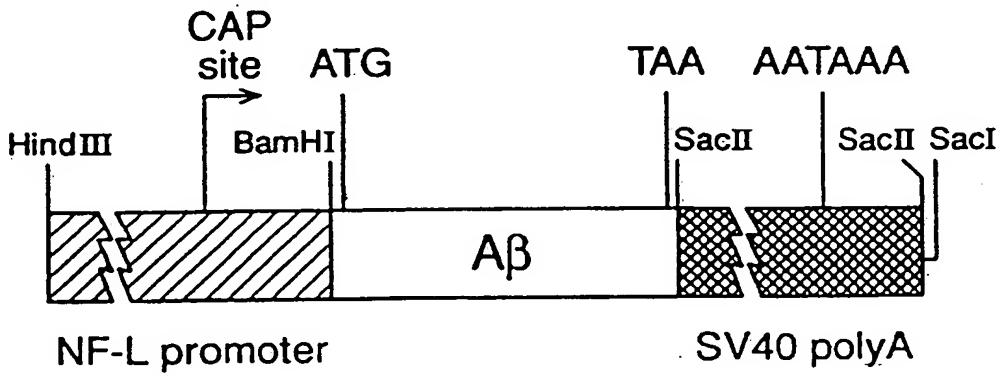
CAP
site
ATG
TAA
AATAAA
HindIII
BamHI
SacII
SacII
SacI
Aβ
NF-L promoter
SV40 polyA

FIG. 1

1 2

FIG. 2

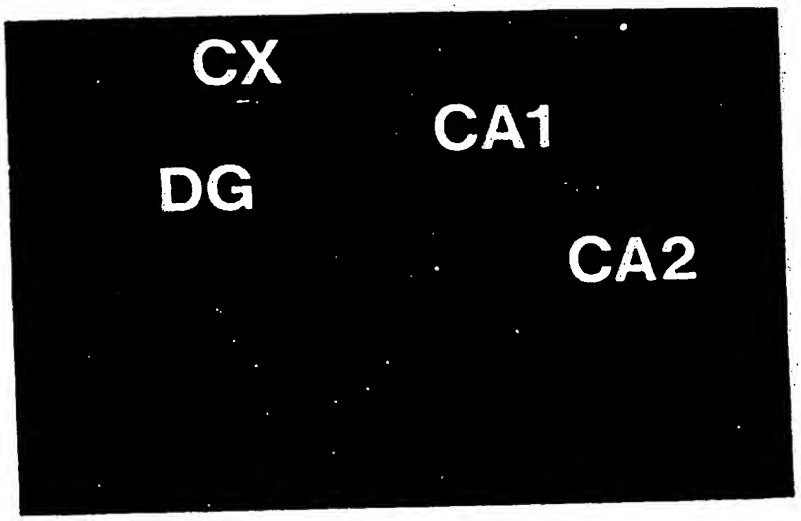

FIG. 3a

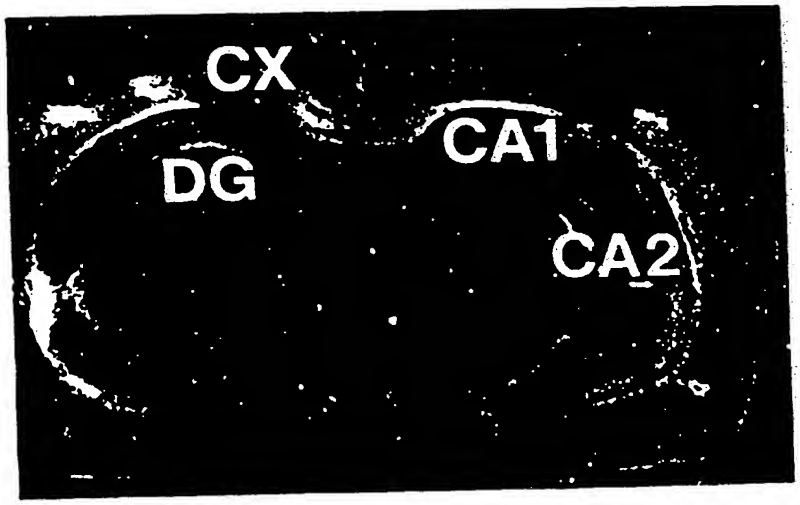

FIG. 3b

FIG. 4a

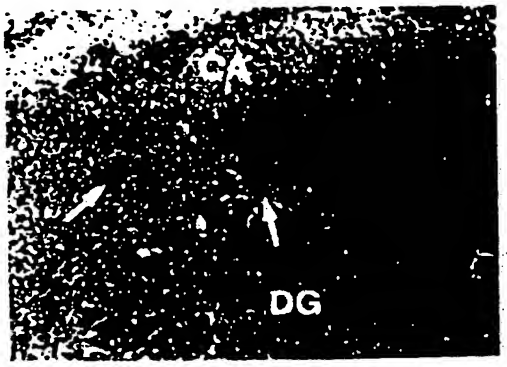

FIG. 4b

FIG. 4c

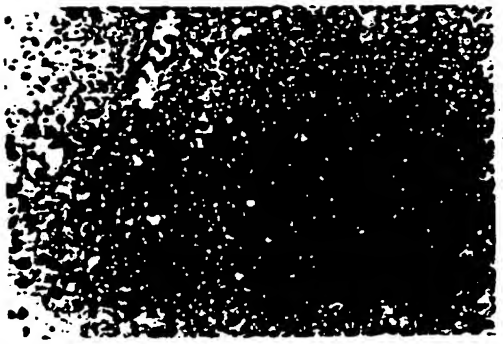

FIG. 4d

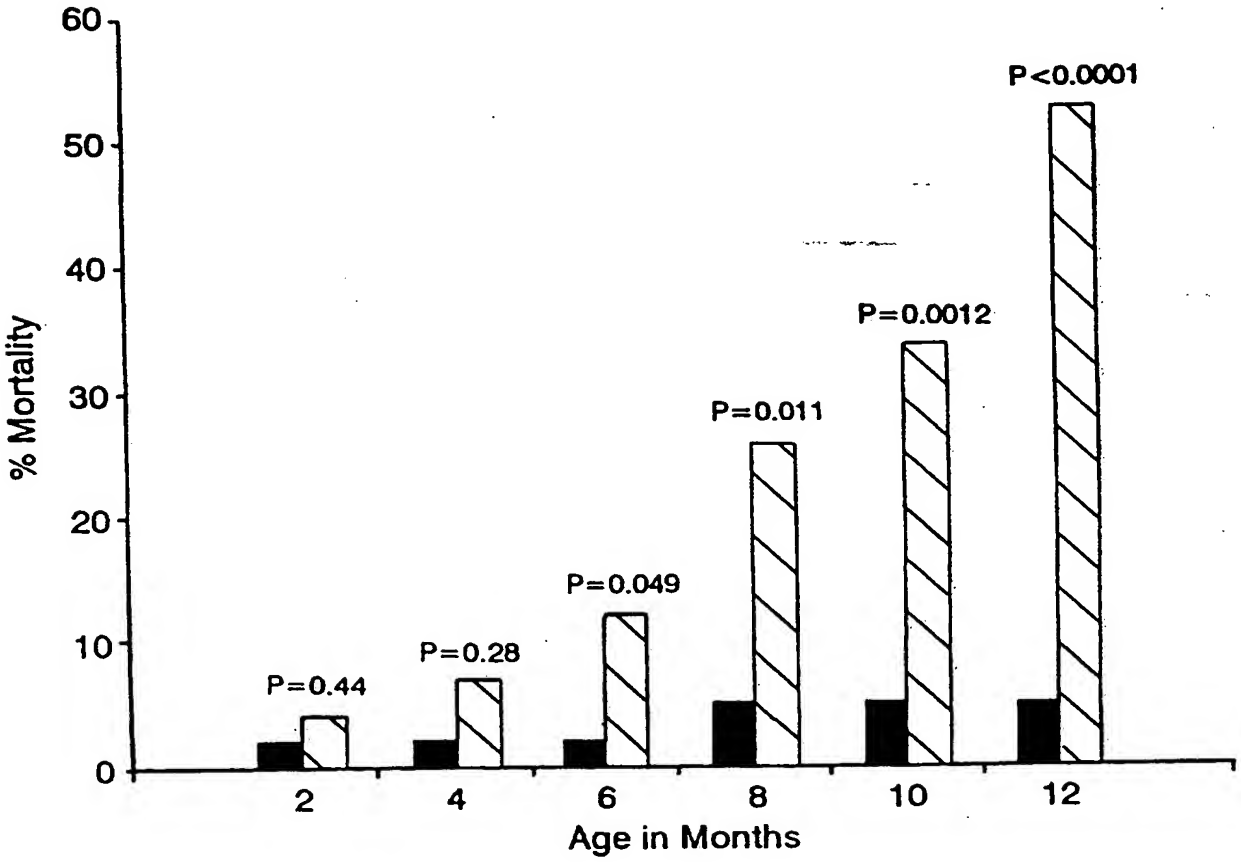
60
50
40
30
20
10
0
% Mortality
P=0.44
P=0.28
P=0.049
P=0.011
P=0.0012
P<0.0001
2
4
6
8
10
12
Age in Months

FIG. 5

FIG. 6a

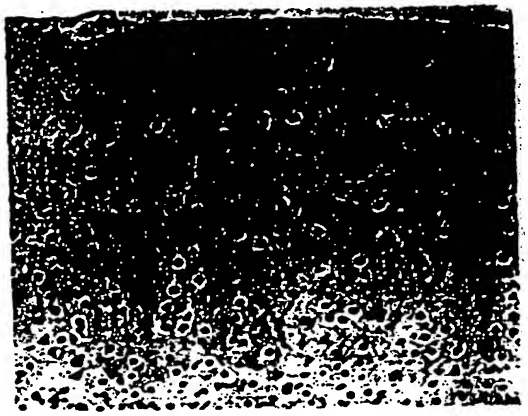

FIG. 6b

FIG. 6c

FIG. 6d

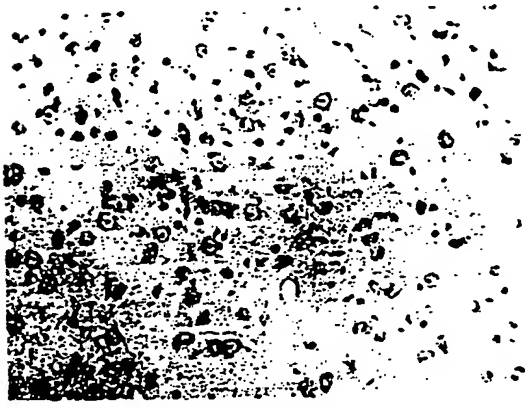

FIG. 6e

FIG. 6f

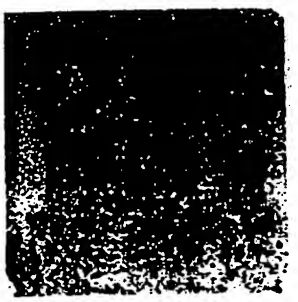

FIG. 7e

FIG. 7d

FIG. 7c

FIG. 7b

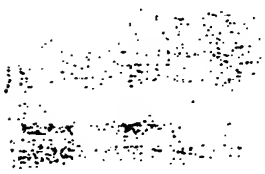

FIG. 7a

FIG. 7h

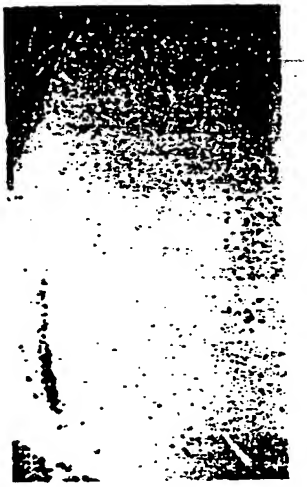

FIG. 7g

FIG. 7f

FIG. 7i

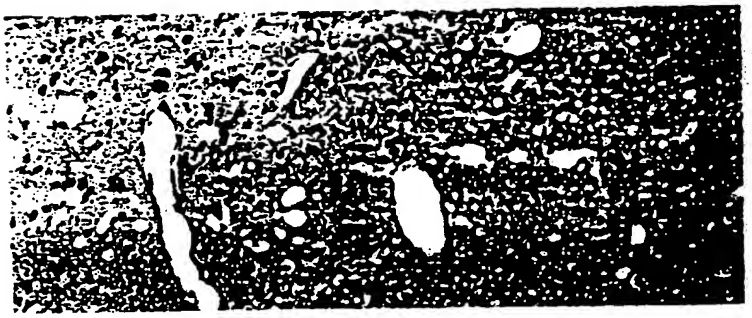

FIG. 7j

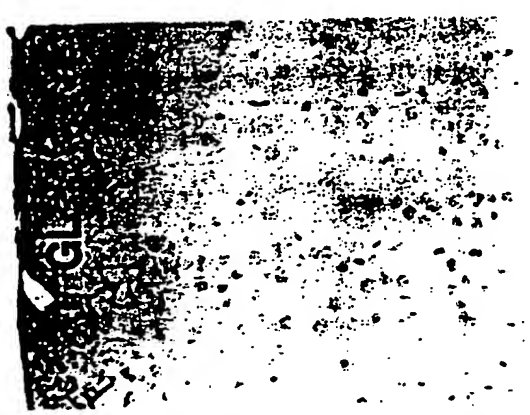

FIG. 8c

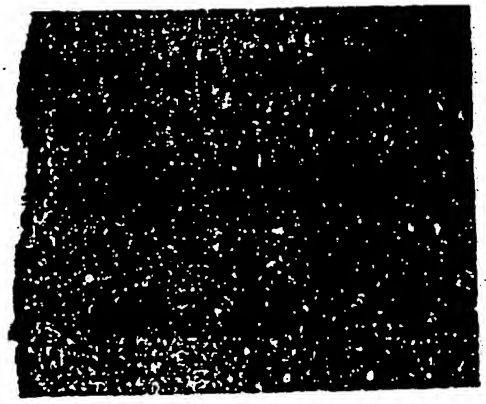

FIG. 8f

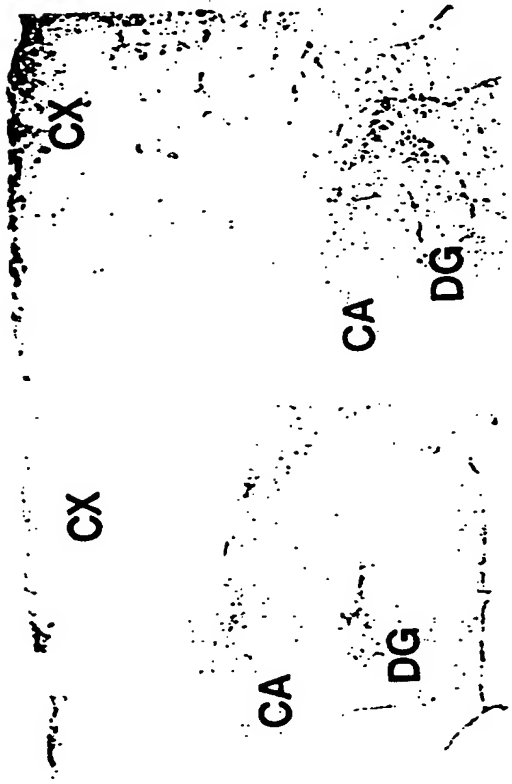

FIG. 8b

FIG. 8e

FIG. 8a

FIG. 8d

```
                1   5    10 13       22                   42
A. Human        DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
B. Murine       ----G----F--R-----------------------------
C. HCHWA-D      ---------------------Q--------------------
```

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13861

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/00; C12N 5/00, 15/00; A61K 49/00
US CL :800/2; 435/172.3, 240.2, 320.1; 536/23.1; 424/9

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 435/172.3, 240.2, 320.1; 536/23.1; 424/9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemical and Biophysical Research Communications, Volume 149, No. 2, issued 16 December 1987, No. 2, T. Yamada et al, "Complementary DNA for the Mouse Homolog of the Human Amyloid Beta Protein Precursor", pages 665-671, especially page 668.	1-22
X	Procedings National Academy of Sciences, Volume 89, issued November 1992, A. Kammesheidt et al, "Deposition of β/A4 Immunoreactivity and Neuronal Pathology in Transgenic Mice Expressing the Carboxyl-Terminal Fragment of the Alzheimer's Amyloid Precursor in the Brain", pages 10857-10861, see especially pages 10859-10869.	1-11 and 23-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
23 JANUARY 1996

Date of mailing of the international search report
14 FEB 1996

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13861

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemical and Biophysical Research Communications, Volume 192, No. 2, issued 30 April 1993, M. Reeben et al, "Tissue-Specific Expression of Rat Light Neuofilament Promoter-Driven Reporter Gene in Transgenic Mice", pages 465-470, especially pages 469-470.	1-22
X	Journal of Clinical Investigation, Volume 93, issued May 1994, Y. Ogawa et al, "Molecular Cloning of the Complementary DNA and Gene that Encode Mouse Brain Natriuretic Peptide and Generation of Transgenic Mice that Overexpress the Brain Natriuretic Peptide Gene", pages 1911-1921, especially pages 1918-1920.	1-11 and 23-27